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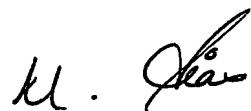
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TITLE OF THE INVENTION (280 characters max)

IMMUNIZATION WITH *Porphyromonas gingivalis* PROTECTS AGAINST HEART DISEASE

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IMMUNIZATION WITH *Porphyromonas gingivalis* PROTECTS AGAINST HEART DISEASE

Porphyromonas gingivalis, a gram negative, anaerobic bacterium is well accepted as the source of the majority of adult periodontal disease (PD) in humans. Currently there are no vaccines in clinical use for control of adult periodontal disease. Conflicting epidemiological data and case reports hint that adult PD may represent a significant risk factor for cardiovascular disease (CVD) in humans. Our recent studies demonstrate that hyperlipidemic mice, homozygous negative at the ApoE gene locus (ApoE^{-/-}), when orally challenged with wild type *P. gingivalis*, develop accelerated atherosclerotic plaque. Most important is our observation that immunization with our vaccine to prevent *P. gingivalis*-elicited periodontal disease prior to oral challenge concomitantly reduces atheroma formation in ApoE^{-/-} mice orally challenged with *P. gingivalis* to levels observed in the unchallenged control ApoE^{-/-} mice.

Thus, the invention is directed to the use of a vaccine against *P. gingivalis* to reduce the risk of CVD in humans that are at "high risk" for developing PD and CVD. We have shown that prophylactic immunization with such a vaccine prevents *P. gingivalis*-accelerated atheroma development in hyperlipidemic ApoE^{-/-} mice. The tested vaccine was a preparation of heat-killed *P. gingivalis* suspended in phosphate buffered saline and consisted of all antigens present as the result of the heat-killing process. Adjuvant was not included in the vaccine and was not used during these studies. Our initial studies show that through immunization with our vaccine candidate to prevent *P. gingivalis* oral infection and subsequent oral bone loss, we reduce the ability of *P. gingivalis* to accelerate atherosclerotic plaque formation. This vaccine provides a method of controlling one of the significant risk factors for CVD, *P. gingivalis* oral infection. Several groups of patients could be helped significantly by this vaccine including: 1- patients with a familial history of both PD and CVD, 2- patients with clinical signs of developing PD that already possess a known risk factor for CVD such as high cholesterol levels, and 3- other "high risk" groups that have been shown to develop accelerated PD and CVD, such as diabetics. The vaccine in its current form has demonstrated prophylactic efficacy in a rodent model of accelerated atherosclerosis, as mice immunized prior to *P. gingivalis* oral challenge were protected from *P. gingivalis*-accelerated atheroma development. This supports prophylactic use of this vaccine. As it is unknown whether PD precedes or follows CVD, we envision that a vaccine against *P. gingivalis* may also possess therapeutic usefulness and provide the benefit of halting the effects of PD on the developing CVD condition.

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1 **The Cardiovascular Disease Periodontal Disease Link:**
2 **Common Theme of Control Through Immunization**

3
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1 Abstract

2 Epidemiological studies support a clinical connection between periodontal
3 disease, a chronic inflammatory disease of the supporting tissues of the teeth, and
4 cardiovascular disease. To test this connection, hyperlipidemic ($ApoE^{-/-}$) mice were
5 orally challenged with *Porphyromonas gingivalis*, the principal bacterium associated with
6 adult periodontal disease, or an invasion-impaired *P. gingivalis* fimbriae-deficient mutant
7 (FimA-). Challenge with wild type *P. gingivalis*, but not with the FimA- mutant, induced
8 oral bone loss, expression of toll-like receptors-2, and -4, and accelerated atherosclerotic
9 plaque formation in $ApoE^{-/-}$ mice. Immunization of $ApoE^{-/-}$ mice to inhibit *P. gingivalis*
10 oral infection concomitantly prevented *P. gingivalis*-accelerated atherosclerosis.

1 **Body of Manuscript**

2 Cardiovascular disease (CVD) is one of the primary causes of death in the
3 western world (1), and despite identification and thorough study of several important
4 factors that predispose humans to CVD including diet, metabolism, exercise, and
5 genetics, an incomplete picture of the etiology of CVD is evident as these factors alone
6 fail to completely predict cardiac events (2). A more thorough understanding of the
7 mechanisms underlying the pathogenesis of CVD has emerged due in part to the use of
8 gene-targeted animals such as the ApoE knockout (ApoE^{-/-}) mouse that develop severe
9 hyperlipidemia, and accelerated atheroma formation (3). Recent investigations have
10 established the importance of inflammation as a determining factor for development of
11 CVD, with emphasis on a mononuclear cellular infiltrate (4), the host response to ox-
12 LDL (5), cytokines (6), C-reactive protein levels (7), cell adhesion molecule expression
13 (8), and identification of Toll-like receptor (TLR)-4 at the site of atherosclerotic plaque
14 accumulation (9). **TLRs are an emerging group of pattern recognition molecules that**
15 **mediate the innate host response to microbial infection (10), and recent reports**
16 **demonstrate that TLRs are selectively up-regulated following infection, as part of**
17 **the host innate response (11, 12).** Due to the reported parallels of several important
18 CVD markers with the host response to infectious diseases, an infectious etiology
19 component to CVD has been suggested (13). It has been reported that *Chlamydia*,
20 *Helicobacter*, Herpes simplex virus, and Cytomeglovirus infection may be linked to CVD
21 (14, 15). However, the connection between infectious diseases, and initiation and/or
22 acceleration of CVD remains speculative due to conflicting reports (16).

1 Periodontal disease, a chronic inflammatory disease of the periodontium that
2 leads to erosion of the attachment apparatus and supporting bone for the teeth (17), is one
3 of the most common chronic infectious diseases of humans (18). *Porphyromonas*
4 *gingivalis* is the primary etiologic agent of adult periodontal disease (19, 20).
5 Epidemiological data, and case reports suggest that *P. gingivalis* oral infection represents
6 a significant risk factor for CVD (21, 22); however, other reports do not confirm the
7 clinical connection between periodontal disease, and increased risk for CVD (JAMA
8 REF) (23). Haraszthy *et al.* (24) recently detected *P. gingivalis* in human atheromatous
9 tissue by polymerase chain reaction, indicating that *P. gingivalis* present in the oral cavity
10 gains access to the vasculature. These results suggest that bacteremia followed by
11 subsequent attachment and invasion of the vascular endothelium by *P. gingivalis* may be
12 responsible for localization of *P. gingivalis* to this site. Experimental studies further
13 support the hypothesis that *P. gingivalis* can aggravate CVD as *P. gingivalis* bacteremia
14 (25), systemic dissemination of *P. gingivalis* with extra-oral infection (26), and injection
15 of *P. gingivalis* into blood vessels of mice stimulates atheroma formation (27).
16 Additionally, *in vitro* studies have reported that *P. gingivalis* invades human endothelial
17 cells (28), elicits cell adhesion molecules (29), and chemokines (30), while a *P. gingivalis*
18 fimbriae-deficient mutant was significantly impaired in invasion, and failed to activate
19 cells *in vitro* (28, 29, 30). The importance of fimbriae for *P. gingivalis*-elicited oral
20 disease pathogenesis has been confirmed *in vivo*, as a *P. gingivalis* fimbriae-deficient
21 mutant failed to elicit oral bone loss using a rat oral infection model (31).

22 Based on these observations, we hypothesized that oral infection of
23 hyperlipidemic ApoE^{-/-} mice with *P. gingivalis*, in a manner that elicits oral bone loss

1 (32), would stimulate accelerated atherosclerotic plaque accumulation. Pilot experiments
2 were initially performed to determine if oral infection of hyperlipidemic ApoE^{-/-} mice
3 with wild type *P. gingivalis* strain 381 leads to dissemination of *P. gingivalis* (escape
4 from the oral environment), as well as detection of *P. gingivalis* in the aortic tissues
5 (localization of *P. gingivalis* in the tissues associated with accelerated atheroma
6 formation). Groups of C57BL-6, and ApoE^{-/-} mice, placed on a normal chow diet, were
7 challenged orally (32) over a 3-week period (n=15 challenges) with either wild type *P.*
8 *gingivalis* strain 381, or an insertional mutant deficient in major fimbriae *P. gingivalis*
9 strain DPG3 (FimA-). This mutant is impaired in attachment, and invasion of epithelial,
10 and endothelial cells *in vitro* (28), fails to elicit inflammatory markers from these cells *in*
11 *vitro* (29, 30), and is not a potent stimulator of oral bone loss (31). We included groups
12 of unchallenged C57BL-6, and ApoE^{-/-} mice to serve as age-matched control populations.
13 Blood samples were obtained from ApoE^{-/-} mice 2 h after the final oral challenge with
14 wild type or mutant *P. gingivalis*, and the aortic arches were obtained 24 h after the final
15 oral challenge. Both wild type *P. gingivalis* and the FimA- mutant were detected in the
16 blood of ApoE^{-/-} mice by PCR amplification of the *P. gingivalis* 16S rRNA gene (Fig.
17 1A) (33, 34). Additionally, PCR amplification of *P. gingivalis* 16S gene from ApoE^{-/-}
18 mouse aortic arch tissue revealed that both wild type, and mutant *P. gingivalis* were
19 present in these tissues (Fig. 1B).

20 To directly test the hypothesis that *P. gingivalis*-elicited periodontal disease
21 accelerates atherosclerotic plaque accumulation, groups of 6-week-old, male, C57BL-6
22 and ApoE^{-/-} mice were placed on a normal chow diet, and were challenged orally with
23 either wild type *P. gingivalis*, or the *P. gingivalis* FimA- mutant, as described above. All

1 animals were sacrificed 6-weeks after the final *P. gingivalis* oral challenge, and were 17-
2 weeks of age (35). As anticipated, C57BL-6 mice, either unchallenged or orally
3 challenged with either strain of *P. gingivalis*, possessed low cholesterol and triglyceride
4 levels (data not shown). Unchallenged ApoE^{-/-} mice possessed high cholesterol and
5 triglyceride levels, and oral challenge with either the wild type or the *P. gingivalis* FimA-
6 mutant had no effect on the serum levels of these molecules, demonstrating that *P.*
7 *gingivalis* oral infection does not lead to modified serum lipid levels in the host (Fig. 2A).
8 Analysis of sera for total *P. gingivalis*-specific IgG revealed no significant differences in
9 the adaptive host response of ApoE^{-/-} mice following challenge with wild type *P.*
10 *gingivalis*, or the FimA- mutant (Fig. 2B). No differences were observed in the ability of
11 wild type *P. gingivalis* to elicit oral bone loss in C57BL/6 or ApoE^{-/-} mice (data not
12 shown). Additionally, we observed that both C57BL/6, and ApoE^{-/-} mice orally
13 challenged with the *P. gingivalis* FimA- mutant did not exhibit significant bone loss (data
14 not shown), and this later observation is in agreement with previous studies reported in a
15 rat model (31).

16 To determine if *P. gingivalis* oral infection leads to accelerated atheroma
17 formation, the aorta of each mouse was harvested from the aortic valve to the iliac
18 bifurcation and morphometric, and immunohistochemical analyses were performed (27,
19 36). It is well established that the accumulation of atherosclerotic plaque on the intimal
20 surface of ApoE^{-/-} mouse aortas occurs at the aortic valves and continues into the aortic
21 arch area (15, 37, 38, 39). Based on this, we determined the total amount of
22 atherosclerotic plaque present on the intimal surface from the aortic valves through the
23 arch. *En face* measurements of the Sudan IV stained atherosclerotic plaque accumulation

1 revealed that ApoE^{-/-} mice challenged orally with wild type *P. gingivalis* possessed
2 significantly more atherosclerotic plaque accumulation on the intimal surface of the
3 aortic arch as compared to unchallenged ApoE^{-/-} mice (Fig. 2C and Fig. 2D). However,
4 despite detection of the *P. gingivalis* mutant in the blood 2 h after oral challenge, and in
5 aortic arch tissue 24 h after oral challenge, ApoE^{-/-} mice challenged orally with the FimA-
6 mutant failed to accelerate atheroma development, as the level of deposited
7 atherosclerotic plaque resembled unchallenged animals (Fig. 2E and Fig. 2F)(40). We
8 conclude that only fully invasive *P. gingivalis* initiate accelerated plaque accumulation
9 (Fig. 2F), and infer that neither localization of *P. gingivalis* due to capture by the
10 spontaneously developing plaque, nor the presence of *P. gingivalis* as demonstrated by
11 PCR is sufficient to drive accelerated atheroma formation. The observed atherosclerotic
12 plaque accumulation elicited by wild type *P. gingivalis* oral challenge did not progress to
13 the thoracic, or abdominal regions of the aorta of ApoE^{-/-} mice. We believe that the lack
14 of progression of the plaque into these regions of the aorta may be a function of the chow
15 diet, and age of these animals (17-weeks of age) (39, 41).

16 Since only the wild type *P. gingivalis* phenotype stimulated accelerated atheroma
17 formation, and we did not observe differences in the adaptive immune response to wild
18 type *P. gingivalis* or the mutant, we hypothesized that differences in the innate immune
19 response to *P. gingivalis* plays a role in *P. gingivalis*-accelerated atheroma formation.
20 Recently, it was reported that the pattern recognition receptor TLR-4, a marker of
21 the innate immune response (13), is up regulated in human, and mouse
22 atheromatous vascular tissues (9). RT-PCR amplification for TLR-2, and TLR-4 in
23 aortic tissue of *P. gingivalis* orally challenged ApoE^{-/-} mice revealed increased expression

1 of TLR-2 and TLR-4 gene transcription in the mice orally challenged with wild type *P.*
2 *gingivalis*. The *P. gingivalis* FimA- mutant challenged mice were negative for TLR-2
3 and TLR-4, and resembled unchallenged animals (Fig. 3A). **TLR-2 and TLR-4**
4 **expression in the aortas of orally challenged ApoE^{-/-} mice was confirmed by**
5 **immunohistochemistry (9, 42).** TLR-2 was detected in aortic tissue of all ApoE^{-/-}
6 mice and was localized primarily at athereromatous lesions (Fig. 3B). Low levels of
7 TLR-2 were observed in aortic tissues of unchallenged mice. Elevated TLR-2 levels
8 were observed in aortic tissue sections from ApoE^{-/-} mice orally challenged with wild
9 type *P. gingivalis*. Slight TLR-2-specific staining was observed in tissue sections
10 from mutant *P. gingivalis* challenged mice. TLR-4 was observed only in the aortic
11 sinus of ApoE^{-/-} mice orally challenged with invasive *P. gingivalis* (Fig. 3B). While
12 aortic tissue from ApoE^{-/-} mice challenged with the *P. gingivalis* FimA- mutant failed
13 to express TLR-4, and resembled unchallenged mice (Fig. 3B). To confirm this
14 observation, we infected primary explant cultures of human aortic endothelial cells to
15 determine if *P. gingivalis* is able to activate TLR expression on aortic endothelial cells *in*
16 *vitro*. We observed that human aortic endothelial cells infected with wild type *P.*
17 *gingivalis* at a MOI of 100 elicited surface expressed TLR-2, and TLR-4 on these cells by
18 FACS, while the FimA- mutant failed to stimulate TLR-2, or TLR-4 expression, and
19 resembled control cells (data not shown; ???SUPPL DATA???). Unexpectedly, we
20 observed that human aortic endothelial cells cultured with the purified FimA
21 protein did not lead to up-regulated expression of either TLR-2 or TLR-4 (data not
22 shown; ???SUPPL DATA???). These *in vitro* and *in vivo* data support that oral
23 challenge of ApoE^{-/-} mice with *P. gingivalis* leads to a host innate immune response to

1 the fully invasive *P. gingivalis*, and fails to initiate an innate host response (as defined by
2 TLR-regulation) in the aortic tissue to the *P. gingivalis* FimA- mutant. Taken together
3 these data indicate that despite initial detection of *P. gingivalis* in the blood, and in the
4 aortic tissue, only wild type *P. gingivalis* infection stimulates an innate immune response
5 as defined by differential TLR expression. This suggests that merely the presence of *P.*
6 *gingivalis* or *P. gingivalis* antigens is not sufficient to initiate and drive accelerated
7 atheroma development as only wild type *P. gingivalis* elicited accelerated atherosclerotic
8 plaque accumulation. Additionally, these data suggest that differences in the innate host
9 response to wild type *P. gingivalis*, as it directly relates to the mechanism of invasion,
10 correlates with the clinical outcome of atheroma formation.

11 Based on our observations that oral challenge of ApoE^{-/-} mice with invasive *P.*
12 *gingivalis* 1- elicited oral bone loss, and 2- accelerated atherosclerotic plaque
13 accumulation, we hypothesized that immunization of ApoE^{-/-} mice to prevent periodontal
14 disease would ameliorate *P. gingivalis*-accelerated atheroma development. To date there
15 are no vaccines in clinical use for prevention of periodontal disease; however, vaccine
16 candidates including heat-killed *P. gingivalis* have been tested in murine models and
17 demonstrate prophylactic control of *P. gingivalis*-elicited oral bone loss (32). To test the
18 hypothesis that immunization to protect animals from oral bone loss, concomitantly
19 prevents accelerated atherosclerosis, groups of ApoE^{-/-} mice (n = 10 mice /group) were
20 immunized twice weekly for 3-weeks with a heat-killed *P. gingivalis* 381 whole
21 organism preparation, without adjuvant, prior to oral challenge with wild type *P.*
22 *gingivalis*. Additionally we included groups of unchallenged, and wild type *P. gingivalis*
23 orally challenged age-matched ApoE^{-/-} mice. Our results confirmed previous data that

1 active immunization of mice with heat-killed *P. gingivalis* elicited a potent *P. gingivalis*-
2 specific serum IgG response (data not shown), and that immunization with heat-killed *P.*
3 *gingivalis* prevented *P. gingivalis*-elicited oral bone loss (data not shown) (32). The *en*
4 *face* morphometric analysis of Sudan IV stained atherosclerotic plaque accumulation on
5 the intimal surface of the aortic arch of ApoE^{-/-} mice revealed that immunization with
6 heat-killed *P. gingivalis* prior to oral challenge protected animals from *P. gingivalis*-
7 accelerated atherosclerotic plaque accumulation (Fig. 4A, B, C, and D). These results
8 demonstrate that by using an effective immunization strategy to prevent periodontal
9 disease, we can completely prevent *P. gingivalis* accelerated atherosclerosis formation.

10 Although conflicting reports exist regarding the ability of infectious diseases to
11 aggravate CVD, this study using wild type *P. gingivalis*, and a defined *P. gingivalis*
12 fimbriae-deficient mutant clearly demonstrate that a specific mechanism by which this
13 bacterial pathogen interacts with the host, and the subsequent host immune response to
14 this pathogen in the aorta, are linked to accelerated atherosclerotic plaque deposition. Of
15 particular interest, we report that immunization to prevent *P. gingivalis*-mediated
16 periodontal disease, also provides the added benefit of significantly reducing the
17 acceleration of CVD. As adult periodontal disease affects an estimated 30% of humans
18 over the age of 35 (18), and cardiovascular disease in one of the major causes of death in
19 the western world (1), further assessment of the interaction of oral pathogens with the
20 host is necessary to develop adequate understanding of the complex processes that link
21 these two significant human diseases.

22 In summary, although CVD is a multifactorial disease, using a combinational
23 approach consisting of a defined genetic mutant of *P. gingivalis*, a site-specific challenge

1 regimen, and immunization studies performed within an effective animal model for
2 assessment of accelerated atherosclerosis, we have demonstrated an experimental link
3 between *P. gingivalis* oral infection and exacerbation of atherosclerotic plaque
4 accumulation in ApoE^{-/-} mice. Our data indicate that the mechanism by which *P.*
5 *gingivalis* accelerates atherosclerosis likely requires bacterial attachment and invasion, as
6 an attachment / invasion mutant failed to accelerate atheroma formation. These data
7 demonstrate that there is a direct impact of the chronic oral disease periodontitis, on the
8 development / acceleration of CVD. Most importantly, our data suggest that vaccination
9 to prevent periodontal disease caused by *P. gingivalis* may concomitantly reduce the risk
10 of accelerated CVD in high-risk groups.

1 **Figure Legends**

2 **Fig. 1.** Following oral challenge, *P. gingivalis* gains access to the circulatory system and
3 localizes in the aortic arch of ApoE^{-/-} mice. (A) PCR amplification of *P. gingivalis* 16S
4 rRNA gene to detect wild type *P. gingivalis* strain 381 (WT), and mutant *P. gingivalis*
5 (FimA-) from blood 2 h after the final oral challenge. (B) PCR detection of *P. gingivalis*
6 in aortic tissue of ApoE^{-/-} mice orally challenged with wild type (WT), or mutant (FimA-)
7 *P. gingivalis* 24 h after oral challenge. Note that both blood and aortic arch tissue are
8 positive for *P. gingivalis* by PCR amplification of the 16S gene. C = control *P. gingivalis*
9 381 DNA (527 bp product), M = 500 bp molecular weight standard, None =
10 unchallenged ApoE^{-/-} mouse.

11

12 **Fig. 2.** Oral challenge of ApoE^{-/-} mice with wild type *P. gingivalis* stimulates accelerated
13 atherosclerotic plaque formation 6-weeks following oral challenge. (A) Serum analysis
14 of total cholesterol, and triglyceride levels from ApoE^{-/-} mice (n = 10 mice/group) at the
15 time of sacrifice (6-weeks following final oral challenge). No statistical differences were
16 observed between ApoE^{-/-} mouse serum levels of total cholesterol, or triglyceride levels
17 in response to wild type *P. gingivalis* (gray bars), or the *P. gingivalis* FimA- mutant
18 (diagonally hatched bars) challenge, and resembled unchallenged levels (black bars)
19 using Student's two tailed t test. Data presented as the mean \pm SD. (B) Assessment of
20 the host adaptive immune response of ApoE^{-/-} mice to *P. gingivalis*. Serum levels of *P.*
21 *gingivalis*-specific IgG was determined by quantitative ELISA prior to oral challenge
22 (blue bars), or at 6-weeks following oral challenge (purple bars). Both wild type *P.*
23 *gingivalis* (WT), and the mutant *P. gingivalis* (FimA-) elicited similar levels of *P.*

1 *gingivalis*-specific IgG ($P>0.9$ by Student's two tailed t test). Data presented as the mean
2 \pm SD. Figures C, D, and E are representative of the atherosclerotic plaque present on the
3 intimal surface of *en face* Sudan IV stained aortic arches of ApoE^{-/-} mice. (C)
4 Unchallenged mouse; (D) mouse orally challenged with the wild type *P. gingivalis*; and
5 (E) mouse orally challenged with the *P. gingivalis* FimA- mutant. (F) Morphometric
6 analysis of the total area of atherosclerotic plaque deposited in the aortic arch of ApoE^{-/-}
7 mice (n = 10 mice/group). ApoE^{-/-} mice orally challenged with wild type *P. gingivalis*
8 (WT) stimulated significantly more atherosclerotic plaque deposition as compared with
9 unchallenged (None), or mutant *P. gingivalis* (FimA-) challenged animals (* = $P<0.05$ by
10 Student's two tailed t test). The mutant failed to elicit accelerated atherosclerotic plaque
11 deposition, and the accumulated plaque resembled unchallenged mice (NS = $P > 0.7$ by
12 Student's two tailed t test). Data presented as the mean \pm SD.

13

14 Fig. 3. ApoE^{-/-} mice orally challenged with invasive *P. gingivalis* express increased
15 TLR-2 and TLR-4 in aortic arch tissue 6-weeks following oral challenge. (A) RT-PCR
16 amplification of TLR-2, and TLR-4 mRNA from aortic arch tissue of either unchallenged
17 (None) ApoE^{-/-} mice, or ApoE^{-/-} mice orally challenged with either wild type (WT), or
18 mutant *P. gingivalis* (FimA-). Unchallenged ApoE^{-/-} mice expressed low levels of TLR-2
19 mRNA, and did not express TLR-4 mRNA. ApoE^{-/-} mice orally challenged with wild
20 type *P. gingivalis* expressed high levels of TLR-2, and TLR-4 mRNA, while mice
21 challenged with the FimA- mutant did not express TLR-2, or TLR-4. (B)
22 Immunohistochemical confirmation of TLR-2, and TLR-4 expression in aortic tissue. As
23 expected, TLR-2 was localized primarily to the sites of atherosclerotic plaque in ApoE^{-/-}

1 mouse aortic arch tissues. Elevated levels of TLR-2, and TLR-4 protein (brown staining)
2 were observed in aortic tissue of $\text{ApoE}^{-/-}$ mice orally challenged with wild type *P.*
3 *gingivalis*. Tissue from the aortic arch of unchallenged $\text{ApoE}^{-/-}$ mice and *P. gingivalis*
4 mutant orally challenged $\text{ApoE}^{-/-}$ mice expressed low levels of TLR-2, and failed to
5 express TLR-4. Irrelevant isotype-matched antibody (IRR-Ab) probed sections were
6 negative for TLR-2 or TLR-4. Original magnification of 100 x for all photomicrographs.

7

8 **Fig. 4.** Immunization of mice with heat-killed *P. gingivalis* prevents *P. gingivalis*-
9 accelerated atherosclerotic plaque accumulation. Figures A, B, and C are representative
10 of the intimal surface of Sudan IV stained atherosclerotic plaque in the aortic arch of
11 $\text{ApoE}^{-/-}$ mice, 6-weeks following oral challenge; (A) unchallenged mouse; (B) animal
12 orally challenged with the wild type *P. gingivalis*; (C) mouse immunized with heat-killed
13 *P. gingivalis* without adjuvant, and orally challenge with wild type *P. gingivalis*. (D)
14 Morphometric analysis of the total area of atherosclerotic plaque deposited on the intimal
15 surface of the aortic arch of $\text{ApoE}^{-/-}$ mice ($n = 10$ mice/group). Mice orally challenged
16 with wild type *P. gingivalis* (WT) possessed significant atherosclerotic plaque as
17 compared to unchallenged (None) mice ($* = P < 0.05$ by Student's two tailed t test).
18 Immunized mice (Immun + WT) were protected from wild type *P. gingivalis*-elicited
19 accelerated atherosclerotic plaque accumulation ($** = P < 0.05$ vs. $\text{ApoE}^{-/-}$ mice orally
20 challenged with wild type *P. gingivalis* by Student's two tailed t test), and resembled
21 unchallenged (None) mice ($NS = P > 0.7$ by Student's two tailed t test). Data presented as
22 the mean \pm SD.

23

1 **Figure S1:** Infection of human aortic endothelial cells with wild type *P. gingivalis*
2 stimulates TLR-2 and TLR-4 expression. FACS analysis of TLR-2 and TLR-4
3 expression on human aortic endothelial cells (Cascade Biologics, Portland, Oregon)
4 cultured with wild type *P. gingivalis* (red trace), or the FimA- mutant (blue trace) at an
5 MOI of 100, and unstimulated cells functioned as controls (shaded black trace). TLR-2
6 and TLR-4 expression was up-regulated on aortic endothelial cells at both 2 and 6 h post
7 challenge. By 24 h TLR expression was not evident and resembled unchallenged TLR
8 expression levels.

9

10

11 **Figure S2:** Human aortic endothelial cells cultured with the purified *P. gingivalis* FimA
12 protein do not express TLR-2 or TLR-4. FACS analysis of TLR-2 and TLR-4 expression
13 on human aortic endothelial cells (Cascade Biologics, Portland, Oregon) cultured with
14 either high dose of *P. gingivalis* FimA (10 μ g/ml; red trace), low dose *P. gingivalis* FimA
15 (1 μ g/ml; blue trace), or were unstimulated (black trace). As a positive control stimulus
16 for TLR-2 and TLR-4 expression, cells were stimulated with wild type *P. gingivalis* at a
17 MOI of 100 (shaded trace). TLR-2 and TLR-4 expression was not evident on aortic
18 endothelial in response to either high or low concentration of the FimA protein, and
19 resembled unchallenged cells, at all time points tested.

20

21

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10

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- 10 34. A 10 μ l sample of whole blood was plated directly on to blood agar plates and
- 11 cultivated anaerobically for up to 10 days. Additionally, 100 μ l of whole blood from
- 12 each mouse was used to detect the presence of the *P. gingivalis* 16S gene by PCR
- 13 amplification (33).. As transient *P. gingivalis* bacteremia are well documented (25), we
- 14 anticipated that blood cultures obtained from ApoE^{-/-} mice challenged with wild type *P.*
- 15 *gingivalis* would be positive. Unexpectedly, all blood cultures were negative for bacterial
- 16 growth.
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1 40. C57BL-6 mice orally challenged with wild type or mutant *P. gingivalis* failed to
2 develop atherosclerotic plaque accumulation on the intimal surface of the aortic arch, the
3 thoracic, or abdominal regions of the aorta, or at any of the furcations, as these mice are
4 resistant to atherosclerotic plaque accumulation when on a normal chow diet.

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6 42. The TLR-2 monoclonal antibody was generated by immunizing Lewis rats with
7 CHO/mouse TLR-2 cells and fusing the spleenocytes to NSO/1 mouse myeloma cells. A
8 rat IgG2b, k antibody clone was chosen based on recognition of CHO/mo TLR-2, and
9 non-reactivity with CHO/hu TLR-2 or CHO/hu TLR-4 by FACS. Nilsen, N., Nonstad,
10 U., Sundan, A., Espevik, T. and Lien, E, manuscript in preparation.

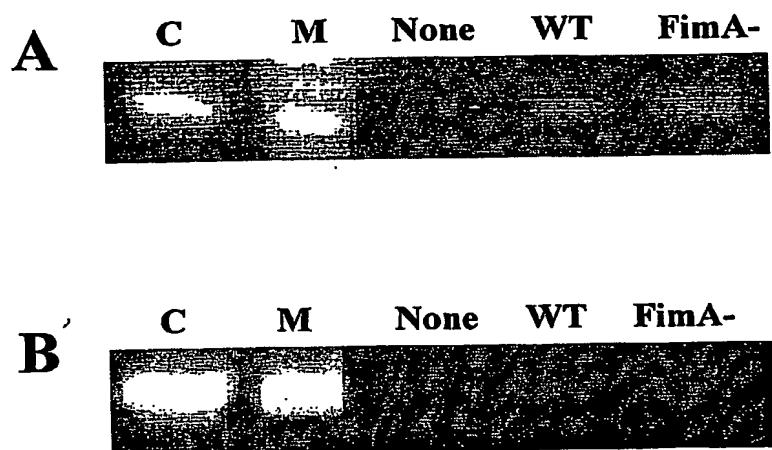
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FIGURE 1



21

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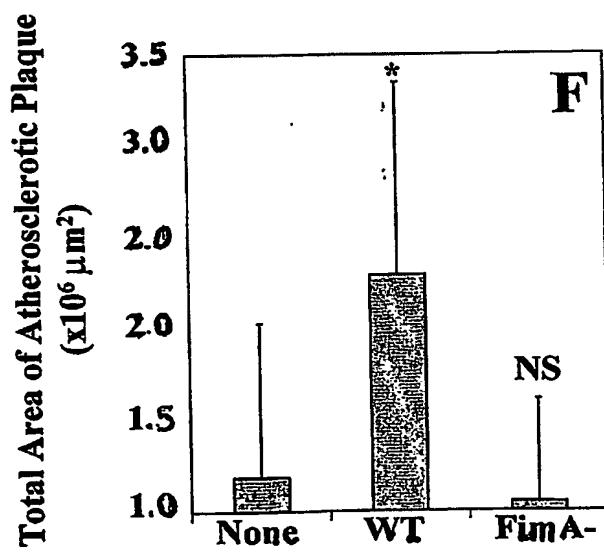
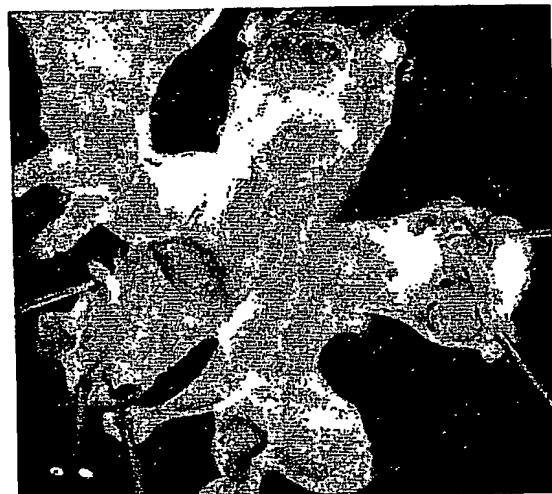
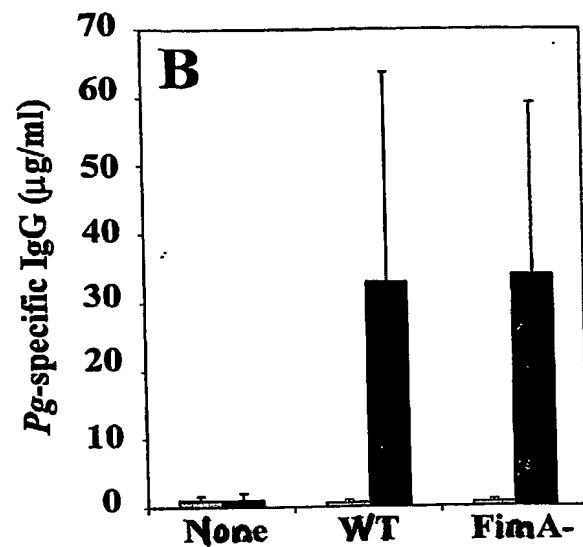
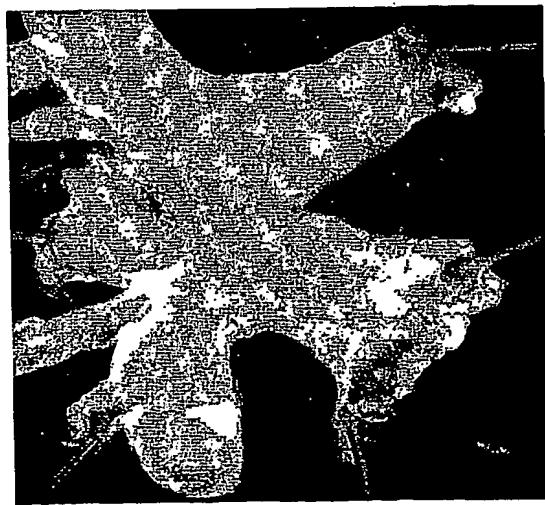
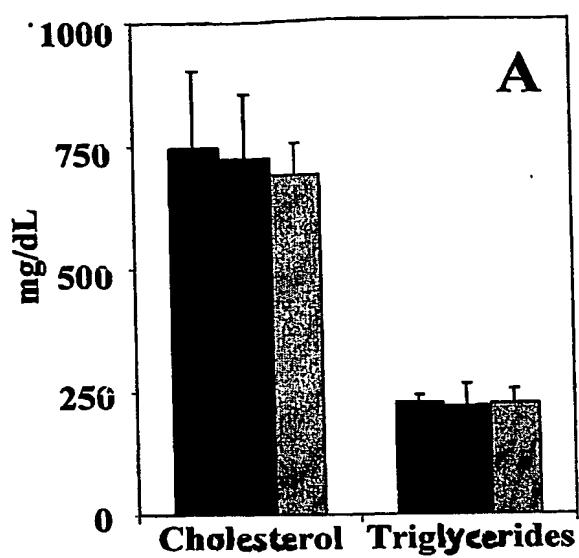


FIGURE 3

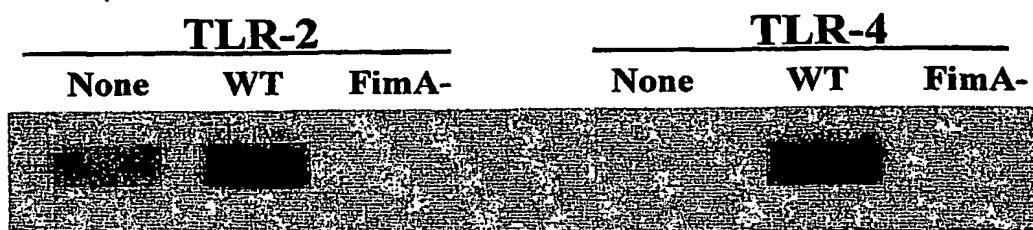
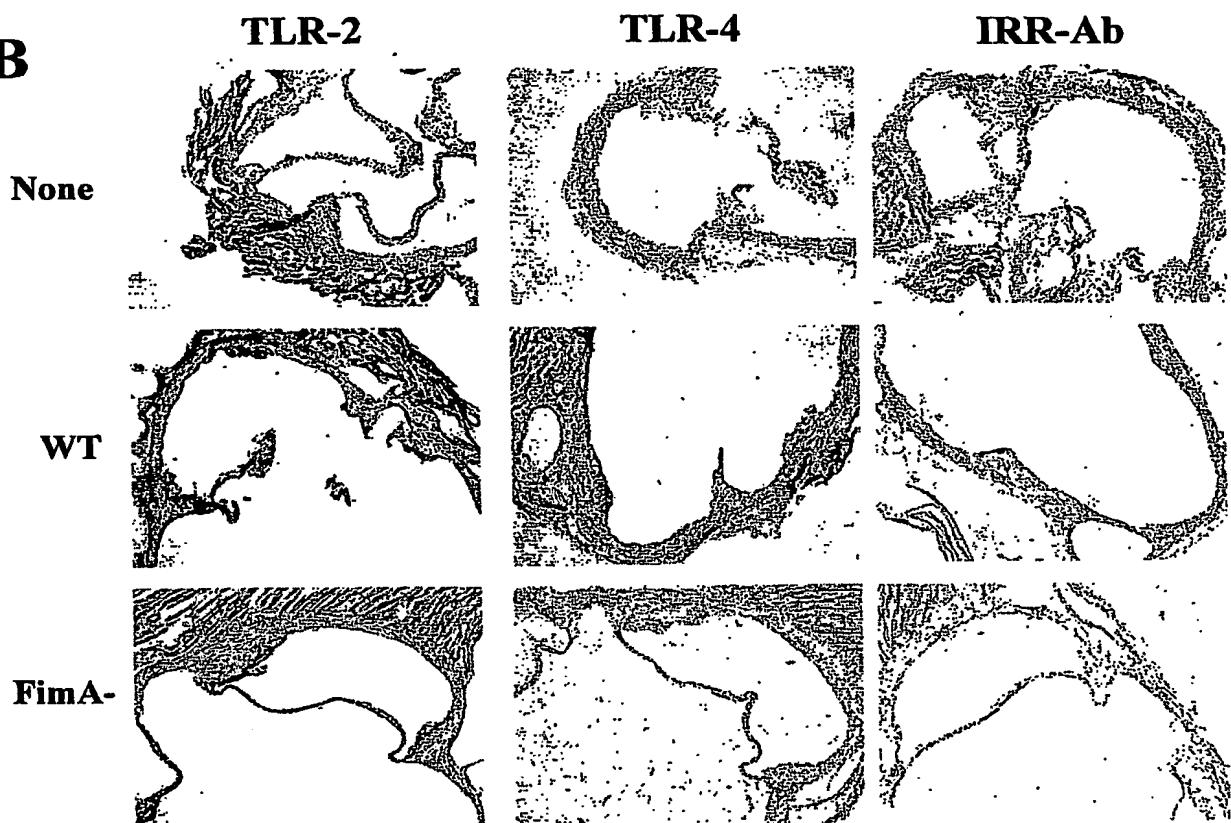
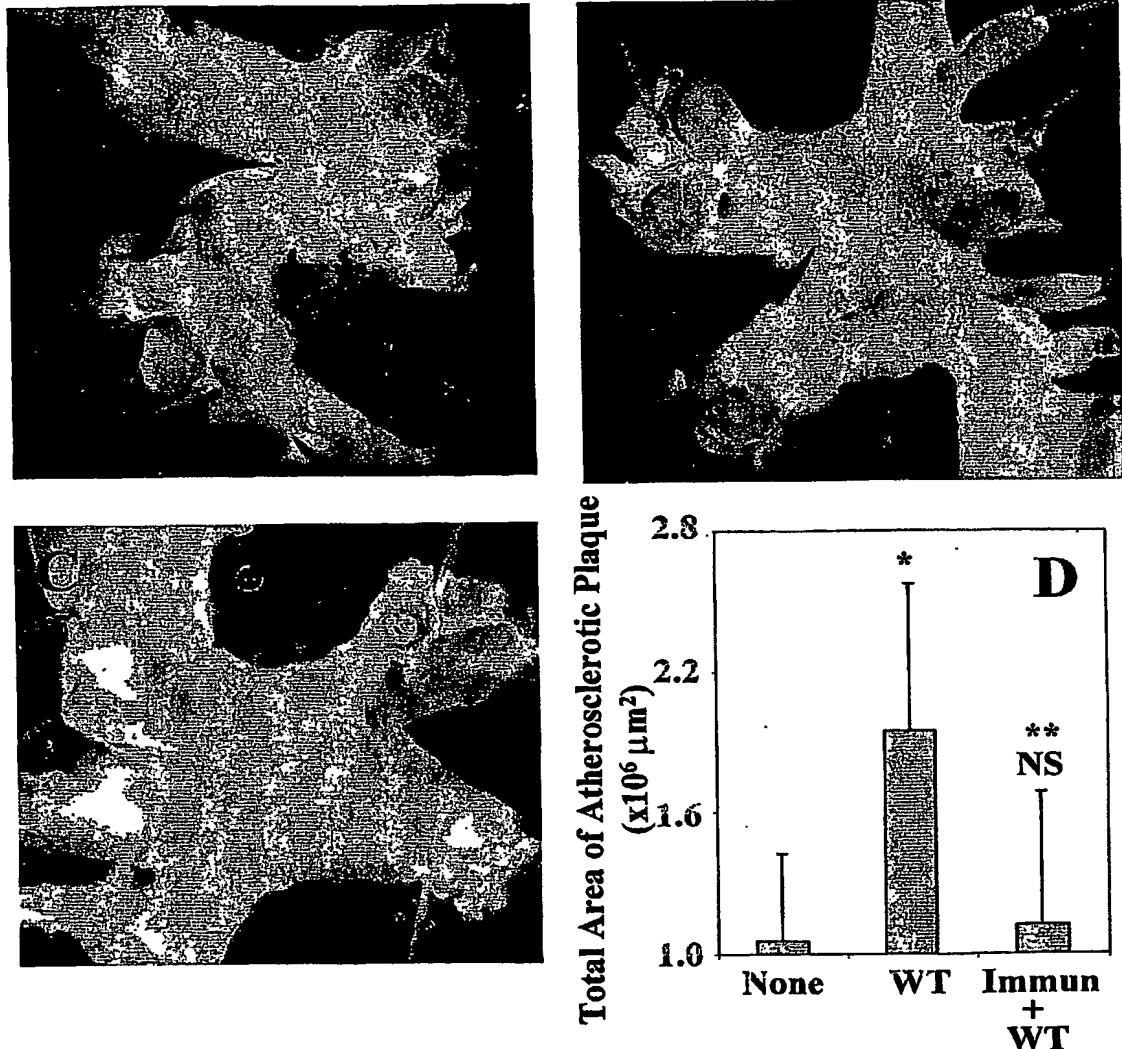
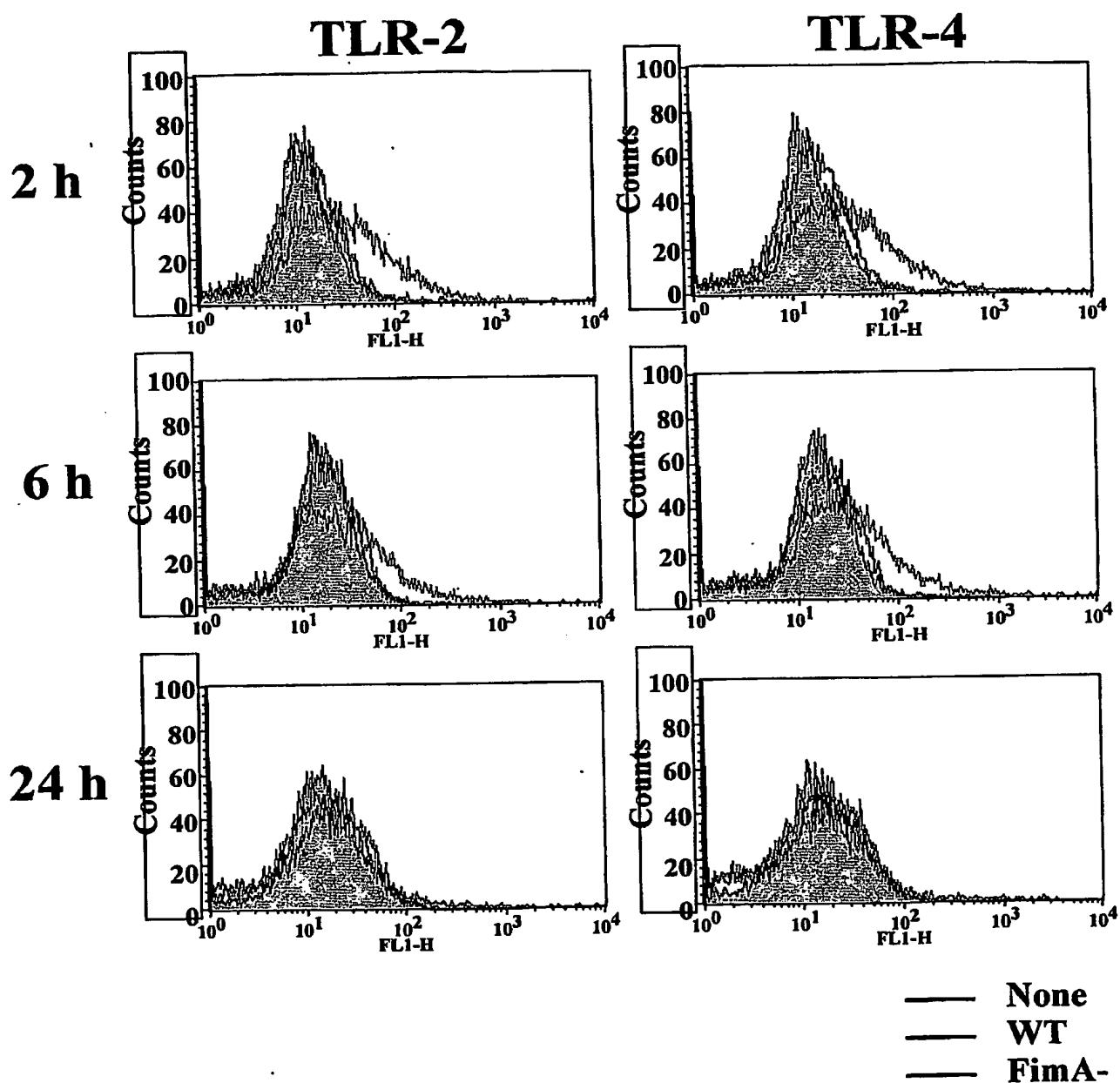
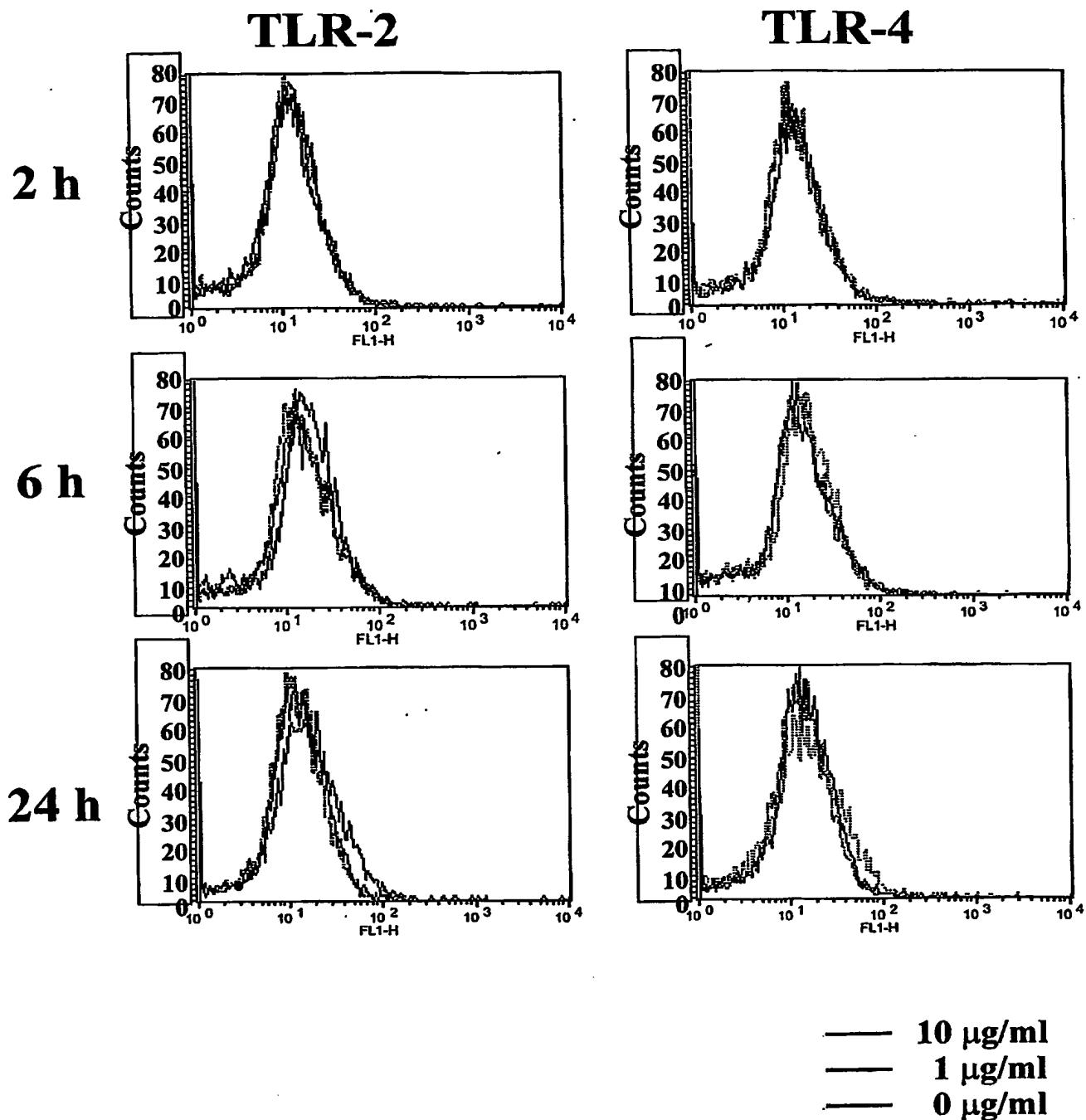
A**B**

Figure 4

Supplemental Data Figure 1
HAEc cells cultured with *P. gingivalis*
Elicit TLR-2 and TLR-4



Supplemental Data Figure 2
HAEc cells cultured with *P. gingivalis*
Fimbriae do not Elicit TLR-2 and TLR-4



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